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THE EFFECTS OF SDF-1 α TREATMENT ON THE MIGRATION OF NEURAL STEM/ PROGENITOR CELLS AFTER TRAUMATIC BRAIN INJURY

Corey Evans

Virginia Commonwealth University

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**THE EFFECTS OF SDF-1 α TREATMENT ON THE MIGRATION OF NEURAL
STEM/PROGENITOR CELLS AFTER TRAUMATIC BRAIN INJURY**

**A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University**

by

COREY J EVANS

Bachelor of Science, Clemson University, 2008

Major Director: Dong Sun, MD, Ph.D.

Department of Neurosurgery

Virginia Commonwealth University

Richmond, Virginia

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List of Abbreviations

ABC	Avidin-biotin complex
BrdU	5-bromo-2-deoxyuridine
CCI	Controlled cortical impact
CDC	Center for Disease Control
CNS	Central nervous system
CSF	Cerebrospinal fluid
CXCR4	C-X-C chemokine receptor type 4
CXCR7	C-X-C chemokine receptor type 7
CXCL12	Chemokine (C-X-C motif) ligand 12
DAB	5, 5-diaminobenzidine
DAR	Department of Animal Research
DCX	Doublecortin
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
GCL	Granule cell layer
GFAP	Glial fibrillary acidic protein
GZ	Granular zone
IACUC	Institutional Animal Care and Use Committee
IC	Injury Cavity
i.p.	Intraperitoneal

LV	Lateral ventricle
MWM	Morris water maze
NIH	National Institute of Health
NPC	Neural progenitor cell
NSC	Neural stem cell
PBS	Phosphate buffered saline
PF	Paraformaldehyde
PNS	Peripheral nervous system
RMS	Rostral Migratory Stream
SCF	Stem Cell Factor
SDF-1 α	Stromal Cell Derived Factor 1- α
Sec	Second
SEM	Standard error margin
SGZ	Subgranular zone
SOX2	SRX (sex-determining region Y) box 2
SSC	Saline sodium citrate
SVZ	Subventricular zone
TBI	Traumatic brain injury
Tuj1	Neuronal Class III β -Tubulin

Abstract

THE EFFECTS OF SDF-1 α TREATMENT ON THE MIGRATION OF NEURAL STEM/PROGENITOR CELLS AFTER TRAUMATIC BRAIN INJURY

By Corey J. Evans

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University

Virginia Commonwealth University, 2010

Advisor: Dong Sun, MD, PhD., Department of Neurosurgery

Traumatic Brain Injury (TBI) is one of the leading causes of death and disability among young adults and has been a significant field in medical research over the past decades. Intensive studies focusing on how to repair tissue damage resulting from head injuries have discovered that the central nervous system (CNS) retains a regenerative capacity throughout life due to the persistent presence of neural stem/progenitor cells (NS/NPCs) in the neurogenic regions. In the normal brain, cells generated in the subventricular zone (SVZ) migrate along the rostral migratory stream (RMS) to the olfactory bulb and cells in the subgranular zone (SGZ) migrate laterally into the granule cell layer of the dentate gyrus. Directed movement of these NS/NPCs is controlled by a variety of factors, and among them the chemoattractant SDF-1 α is of particular importance. Studies have identified that the chemokine SDF-1 α and its receptor

CXCR4 play an important role in guiding cell migration in many types of cells including NS/NPCs. The current study tested if SDF-1 α could be delivered through alginate to attract and guide migration of NS/NPCs and its progeny both *in vitro* and *in vivo*. Using a Boyden chamber migration assay, we found SDF-1 α either added directly in the medium or incorporated into alginate threads was capable of influencing migration of cultured NS/NPCs in a dose-dependent manner. In the *in vivo* study, when injected directly into the cerebral cortex, SDF-1 α showed limited capability in inducing neuroblasts migration off the normal tract to the site of SDF-1 α injection. When SDF-1 α was delivered via alginate thread to the focal injury site at 2 days post TBI, significantly increased number of migrating neuroblasts derived from the SVZ was observed around the injury site. Increased expression of SDF-1 α receptor CXCR4 was observed in the NS/NPCs in the SVZ and around the injury site following TBI. These data suggest that bioactive SDF-1 α can be delivered via alginate thread and exogenous delivery of SDF-1 α and its interaction with receptor CXCR4 mediates migration of newly generated neurons from the SVZ to the site of injury following TBI. Collectively, our study indicates that SDF-1 α could be utilized as a guidance cue for tissue repair following brain injury.

Chapter 1 – Introduction and Background

Traumatic Brain Injury (TBI), one of the leading causes of death and disability in young people, has become an increasingly significant field in medical research over the past decade. Intensive clinical studies focusing on how to reverse damage resulting from head injuries have discovered that the central nervous system (CNS) appears to retain some level of regenerative capacity throughout life due to the presence of neural stem/progenitor cells (NS/NPCs), which persist in the CNS and allow potential regeneration of the damaged neural tissue lost following injury. Researchers have found that TBI increases proliferation of these neural stem cells. Furthermore, the degree of injury-enhanced neurogenesis is age-dependent with much higher level of cell proliferation and generation of new neurons in younger brain than the older one, providing a possible explanation for clinical findings that younger patients following TBI have a better chance for cognitive improvement over time.

NS/NPCs are found in two distinct regions in the CNS: the subventricular zone (SVZ) in the wall of the lateral ventricle and the dentate gyrus (DG) of the hippocampus. NS/NPCs are most active during neural development but retain the ability to proliferate and can differentiate into neurons and glial cells throughout life. In the normal brain, stem cells generated in the SVZ migrate along the rostral migratory stream (RMS) to the olfactory bulb and cells in the subgranular zone (SGZ) migrate laterally into the granule cell layer of the DG. Directed movement of these NS/NPCs is controlled by a variety of factors, notably the chemoattractant SDF-1 α , which is often cited as an important regulatory chemokine in both the RMS and the DG. After brain injuries such as TBI, stroke, and other ischemic conditions, the degree of proliferation of NS/NPCs are increased in both the SVZ and the DG. Studies have also found an increased migration of newly generated neurons from the neurogenic regions to the site of injury

particularly ischemic injury and this migration is linked to local release of SDF-1 α by the damaged tissues. As SDF-1 α and its receptor CXCR4 interaction can act as guidance cue for migration of newly generated neurons, this study will test if it could be utilized to attract and guide TBI-enhanced newly generated neurons to the site of injury to replace damaged neurons following TBI. This thesis will first focus on the characteristics of the injury and animal models used. The delivery method of SDF-1 α and its effect of inducing cell migration both *in vitro* and *in vivo* will be discussed.

Traumatic Brain Injury in the United States

Traumatic Brain Injury (TBI) is a major healthcare concern both in the United States and around the world. Although approximately 1.5 million Americans sustain some form of TBI each year, the mechanisms and resultant complications of TBI are still not well understood. TBI results in 50,000 fatalities and 250,000 other severe injuries in a given year, making it one of the leading causes of death and long-term disability in the U.S. (Langlois et al., 2006). Although advances in treatment and management of TBI have resulted in a decrease in the number of TBI deaths, it has consequently led to increased numbers of patients suffering the long term effects of TBI. Currently an estimated 5.3 million Americans are living with some form of TBI-related disability, often having lifelong effects on both patients and their families. In other developing countries, the rate of incidence of TBI can be even higher. The total financial cost of TBI and injury-related care in the U.S. is also estimated to be approximately \$60 billion per year (Langlois et al., 2006).

Pathology of TBI

Typical TBI occurs in one of 3 ways: focal injury, often caused by a collision of the skull with a hard object; diffuse injury, such as a rotational injury that may be experienced during a car accident; and a combination of both focal and diffuse injuries. The direct injuries caused, known as the primary insult, is the beginning of a biphasic process of cascading damage that can last for anywhere from a few hours to a few weeks (Dutton and McCunn, 2003; Davis, 2000). The primary insult is the immediate injury due to mechanical forces and can result in tissue damage, contusion, hemorrhage, and diffuse axonal injury. This primary insult will give way, over the course of hours to days, to a series of delayed non-mechanical events known as the secondary

insult. This secondary damage consists of inflammation, excitotoxicity, ischemia, cerebral edema, secondary axotomy, and delayed cell death, the combination of which results in a multitude of long term physiological problems for the patient (McIntosh et al., 1996).

Experimental TBI

To study the pathophysiological changes following TBI and evaluate therapeutic strategies for TBI, several varying injury models have been employed to simulate TBI in animals, and the most widely used are Controlled Cortical Impact (CCI) and Fluid Percussion Injury (FPI) models. Specifically, CCI is very effective in reproducing the primary and secondary insults that would commonly be seen in a focal brain injury (Dixon et al., 1991). This method uses a pneumatically driven impactor to produce TBI and results in substantial cortical tissue loss at the impact site as well as neuronal cell loss in several regions of the hippocampus. Primary damage is also common along the corpus callosum and may also lead to breakdown of the blood-brain barrier. Another attribute of the CCI model is the formation of a medium-sized injury cavity in the cortical tissue several days after injury, which is an important factor in our introduction of SDF-1 α to cortical tissue (Smith et al., 1995).

Neural Stem Cell Proliferation and Neurogenesis

A long-held belief within the scientific community suggested that new neurons could not be generated in the CNS following initial development. Researchers eventually found that two areas of the mature mammalian brain, the subventricular zone (SVZ) of the lateral ventricle and the dentate gyrus (DG) of the hippocampus, continually generate new neurons throughout life (Altman and Das, 1965; Lois and Alvarez-Buylla, 1993). In each location, neural stem cells give

rise to neuroblasts, which have the ability to differentiate into both neuronal and glial cell types and migrate to their target regions (Figure 1.1).

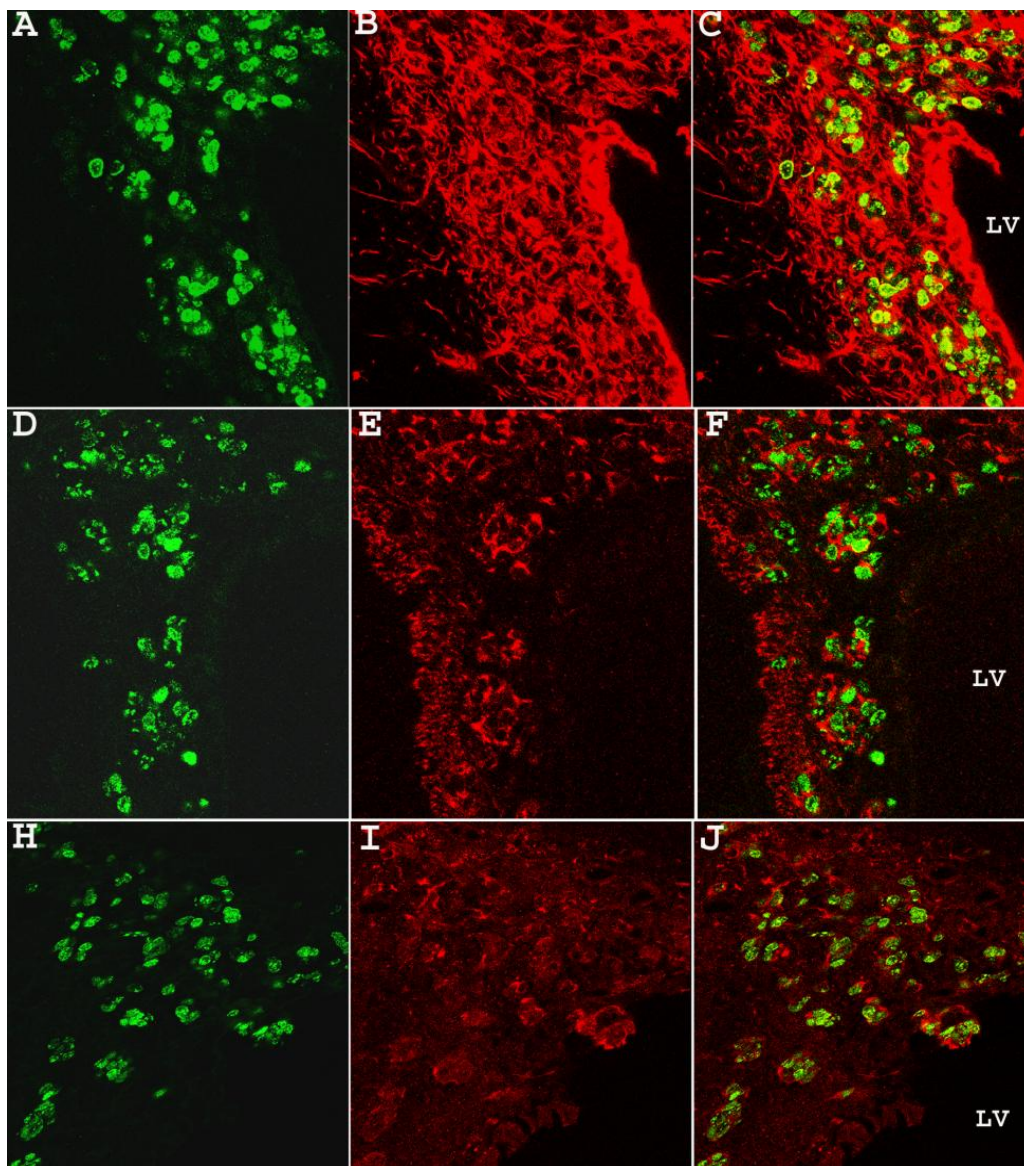


Figure 1.1: Differentiation of NS/NPCs in the SVZ. Confocal photographs show the upper regions of the SVZ in the lateral ventricle of an adult, uninjured rat. Images A, D, and H show NS/NPCs labeled with BrdU (green) which labels newly generated cells. Image B shows cells stained with GFAP (red), a marker for neural stem cells and astrocytes. Images E and I show cells labeled with Tuj1 and Toad 64 (both red), respectively, both are markers for immature neurons. Images C, F, and J are overlay images of A&B, D&E, H&I, respectively.

Subventricular Zone (SVZ): Located under the ependymal cells in the lateral wall of the lateral ventricle, the SVZ contains the largest population of dividing neural stem cells in the adult brain (Chirumamilla et al., 2002). The SVZ itself contains three different types of cells: slow dividing B cells, which are the true multipotent neural stem cells; transit amplifying type C cells, which in turn rapidly divide; and type A cells, which are the migratory neuroblasts that are constantly moving out of the SVZ (Burns et al., J Comp Neurol 2009: 515: 125, Figure 1.2). After moving out of the SVZ, these neuroblasts subsequently migrate along the rostral migratory stream (RMS), consisting of a multitude of neuroblasts undergoing a process known as chain migration. These neuroblasts follow migratory cues releases by cells surrounding the RMS and migrate tangentially to the olfactory bulb, where they move radially into the granular and periglomerular layers and finally differentiate into olfactory granule neurons (Doetsch et al., 1997; Garcia-Verdugo et al., 1998).

The second major neurogenic region of the adult brain is the subgranular zone (SGZ), a 2-3 cell layer of a narrow area located between the granular cell layer and the hilus region in the dentate gyrus of the hippocampus. In the SGZ, dividing type B neural stem cells give rise to precursor cells which migrate along the radial projection of type B cells and become dentate granule neurons (Burns et al., 2009; Figure 1.3).

Both of these neurogenic locations contain populations of neural stem/neural progenitor cells, which are the cells that give rise to new neurons during development and throughout life. In theory, the same migratory cues that direct neuroblasts migration to specific areas of the CNS could also be used to direct newly generated neurons to injured areas following TBI.

Adult generated new neurons can integrate into the existing neural circuitry and participate in physiological functions. In normal brain, neurons generated from the SVZ migrate along the rostral migratory stream to the olfactory bulb becoming olfactory interneurons involving fine olfactory discrimination (Gheusi et al., 2000; Enwere et al., 2004) and olfactory perceptual learning (Moreno et al., 2009), whereas in the DG, newly generated neurons from the subgranular zone migrate laterally to the granule cell layer of the DG becoming granular neurons involved in hippocampal-dependent learning and memory function (Shors et al., 2001, 2002; Kempermann and Gage, 2002; Aimone et al., 2009; Jessberger et al., 2009). Under pathological conditions, such as TBI and stroke injury, the level of cell proliferation and neurogenesis increase in both the SVZ and the DG suggests that the brain has an endogenous repair mechanism following brain injury.

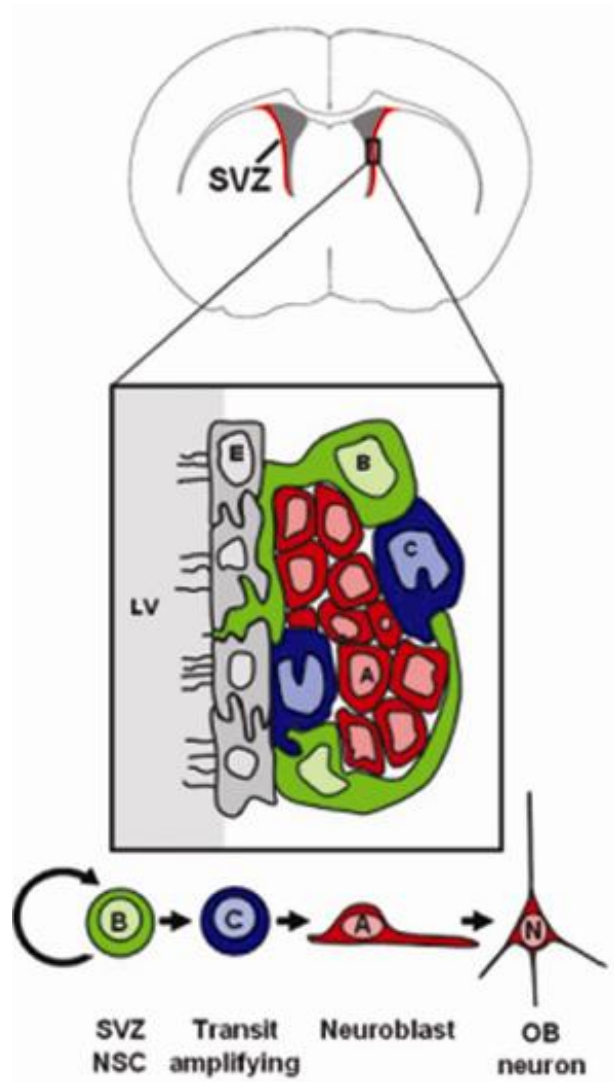


Figure 1.2: NS/NPCs in the SVZ of the lateral ventricle. There are 3 types of cells in the SVZ, including slow dividing B cells which give rise to rapidly dividing (transit amplifying) type C cells. Type C cells in turn give rise to migratory type A neuroblasts which reach olfactory bulb becoming olfactory granule neurons (Burns et al., 2009).

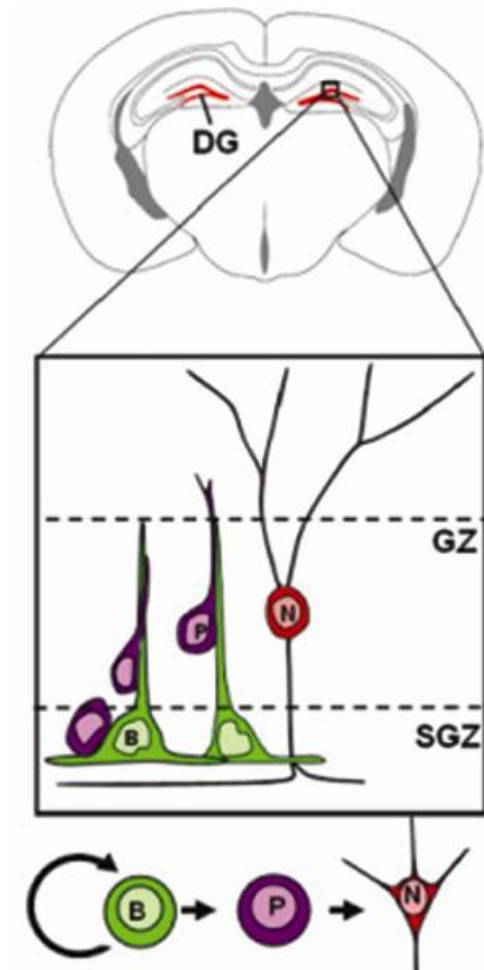


Figure 1.3: NS/NPCs in the SGZ of the dentate gyrus in the hippocampus. In the SGZ, dividing type B cells give rise to precursor cells which migrate up the radial projection of type B cells to become dentate granule neurons (Burns et al., 2009).

Neurogenesis and Brain Injury

In the normal CNS, the adult brain maintains some levels of neural progenitor cell proliferation throughout life. Following certain pathological conditions, these responses are greatly enhanced (Dash et al., 2001; Sun et al., 2005; Gould and Gross, 2002; Temple and Alvarez-Buylla, 1999). Studies have found that TBI is accompanied by a proliferative response in the subventricular zone of the lateral ventricle, the dentate gyrus of the hippocampus, and the injury site itself (Chirumamilla et al., 2002; Sun et al., 2005; Dash et al., 2001). Focal ischemic injury has also been shown to enhance cell proliferation in the SVZ (Parent et al., 2002; Zhang et al., 2001). These studies suggest that the adult brain retains the ability to partially regenerate and repair brain tissue following injury.

Previous studies in our lab have shown that TBI can enhance cell proliferation in both the SVZ and the DG in adult rats, with the most robust response seen at two days post injury (d.p.i.). This injury-enhanced cell proliferation lasts for seven days post injury and gradually returns to sham levels by fourteen days post injury (Sun et al., 2005) (Figure 1.4).

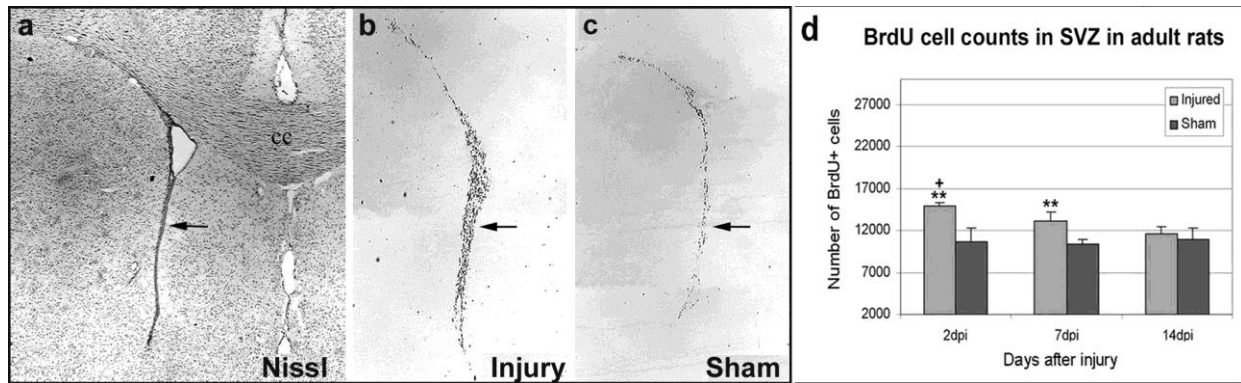


Figure 1.4: TBI-induced neurogenesis in the SVZ of adult rats. a) Nissl staining micrograph showing the SVZ (arrow). b-c) Proliferating cells were labeled with BrdU showing a pronounced proliferative response in an injured animal at 2 days post injury (b) as compared to the level of cell proliferation in a sham brain (c). Graph in d) shows the quantification analysis of the total number of BrdU-positive cells in the ipsilateral SVZ in injured and sham animals. At 2 days post-injury (d.p.i.), the total number of BrdU+ cells is much higher in the injured animal than in the sham. BrdU cell counts are also significantly higher at day 7 post-injury. At 14 days post-injury, BrdU cells counts are slight but not significantly higher in injured animals compared to sham.

Migration of NS/NPCs

The developing and adult CNS both utilize a complex system of neurotransmitters, chemoattractants, and guidance molecules to precisely regulate neuroblast migration (Hagg et al., 2005). Appropriate migration and placement of neurons during development is essential for the construction of functional synaptic circuitries in the brain. For example, radial glia provide a scaffold for directed cell migration in the developing brain and are in turn regulated by reelin, which also functions as a detachment signal (Götz and Huttner, 2005; Rakic, 2003). Other molecules regulating the neuroblast migration include guidance cues such as astrotactin and chemoattractive cues like neuregulin 1 (NRG-Ig) and CXCL12 (SDF-1 α) (Yokota et al., 2007).

In adult brain under normal conditions, newly generated neurons in the SVZ migrate along RMS under unknown guidance cues. In pathological conditions such as stroke injury, diverse chain migration of new neurons from the SVZ to the site of ischemic cortical or striatum regions have been observed. Such ectopic migration of new neurons is believed to be related to the focal release of chemokine SDF-1 α and the upregulation of its receptor CXCR4 expression at the site of ischemic injury (Robin et al., 2006; Kojima et al., 2010). Following TBI, there are limited studies reporting cell migration (Itoh et al., 2009).

Basics of SDF-1 α /CXCL12

Stromal Cell-derived factor 1 α (SDF-1 α), also known as CXCL12, is a small cytokine which belongs to the CXC family of chemokines and binds to the chemokine receptor CXCR4 (Figure 1.5) (Ohtani et al., 1998; Stumm et al., 2002; Tran and Miller, 2003). SDF-1 α is expressed in many tissues such as brain, thymus, heart, and bone marrow, but it is most important for regulating neural stem cell migration in the developing brain (Bagri et al., 2002; Ponomaryov et al., 2000). During neural development, SDF-1 α is released in order to guide the migration of neural stem cells from the external germinal layer into the internal granule layer (Ma et al., 1998; Suzuki et al., 2001; Zou et al., 1998). Neural stem/neural progenitor cells located in the SVZ and SGZ of the adult rat brain express SDF-1 α and CXCR4 (Tran and Miller, 2003). In studies involving stroke and cerebral ischemia, SDF-1 α has been found to be relevant in stroke-induced NSC migration and proliferation (Imitola et al., 2004). Also, SDF-1 α production is upregulated following cerebral ischemia, suggesting SDF-1 α may have a role in NSC proliferation and migration in stroke victims (Hill et al., 2004; Stumm et al., 2002).

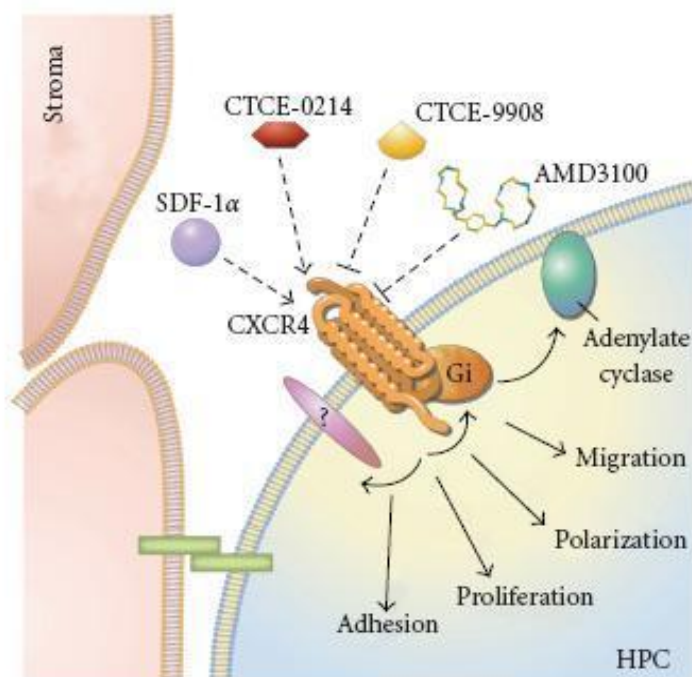


Figure 1.5: CXCR4 receptor. The membrane bound receptor CXCR4, also known as fusin, is the only confirmed receptor for the ligand CXCL12, also known as SDF-1 α . Two of the most important functions of CXCR4 are cell proliferation and migration.

Role of SDF-1 α in NSC Migration

Since neural stem cells expressing CXCR4 have a capacity for precise migration to widespread and distant areas of pathology, the SDF-1 α /CXCR4 interaction has long been looked at as a possible avenue for treatment (Imitola et al., 2004). In regions of the CNS such as the SVZ, NSCs undergo a unique form of tangential migration known as chain migration (Figure 1.6) (Lois et al., 1996). NSCs may use this same mechanism in response to injury, allowing migration of newly generated neurons to areas that are not located in their normal migratory paths. *In vitro* studies have shown that addition of SDF-1 α induces a significant increase in chain migration emanating from neurospheres of NSCs (Imitola et al., 2004). To verify these results, studies have used antibodies that block the actions of SDF-1 α and CXCR4 to test if cell migration was affected in any way. One used a neutralizing antibody against CXCR4 to block SDF-1 α /CXCR4 binding *in vitro* and found that migration was significantly attenuated compared to normal levels (Robin et al., 2006).

Even though the SDF-1 α /CXCR4 relationship has been proven integral to the chain migration process, the mechanism of its upregulation following TBI is still unclear. Recent studies have determined that levels of SDF-1 α mRNA expression and SDF-1 α protein synthesis around the damaged area after TBI did not increase when compared with the corresponding levels in the sham operated cortex (Itoh et al., 2009; Figure 1.7). However, the data has indicated that SDF-1 α is constantly synthesized in normal and injured brains, and TBI may cause leakage of SDF-1 α from surrounding damaged tissue which could, in turn, induce cell migration (Itoh et al., 2009). Since levels of SDF-1 α production are not increased following

TBI, external introduction of SDF-1 α to the injured cortical tissue may be able to influence neuronal migration to some effect.

Use of alginate as a vehicle for SDF-1 α delivery

Alginate is a versatile, naturally occurring polymer that can provide a matrix to ensure gradual, sustained release of a chemokine over a period of several days. It also has the advantage of being biodegradable, which allows implantation of any matrix created with alginate into the body or specifically, the nervous system. Alginates are broken down by proton-catalyzed hydrolysis based on a combination of time, pH, and temperature. As the alginate thread dissolves, the chemokine under investigation is slowly released into surrounding tissue and may influence cells in the area. Since alginates also have the capability for high levels of hydration, they have been used often as vehicles for drug delivery (Tonnesen and Karlsen 2002). For our purposes, the major advantage of using alginate is that it allows sustained release of SDF-1 α in order to provide a constant source of migratory cues to cells.

Alginate threads can be tailor-made to suit a wide variety of applications and are designed to come in a wide variety of shapes and sizes. For our investigations, we prefer to use a rod-shaped alginate thread that measures approximately 3mm long and 1mm wide (Figure 1.8). At this size, the thread is easily introduced into the injury cavity of an animal injured with CCI.

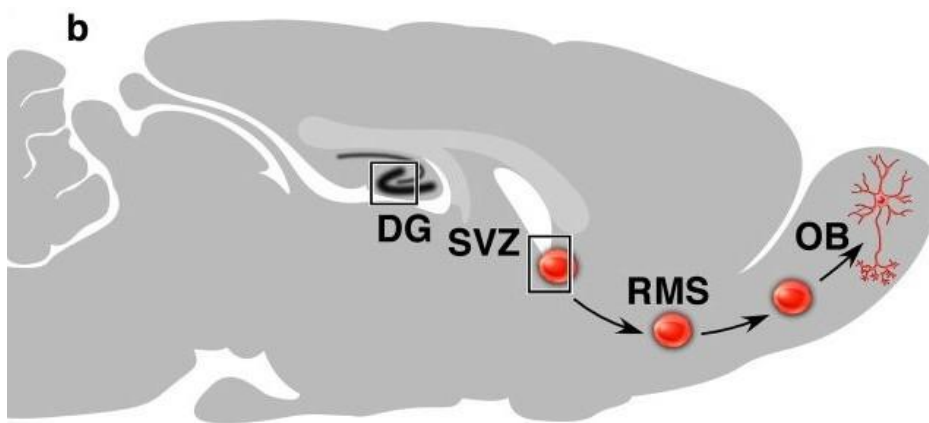


Figure 1.6: Newly generated cells from the SVZ migrate along RMS to the olfactory bulb.

Neuroblasts (type A cells) are derived from proliferating NS/NPCs (type B cells) in the SVZ and then migrate tangentially within the rostral migratory stream (RMS) to the olfactory bulb. Here the neuroblasts are able to differentiate into olfactory granule neurons.

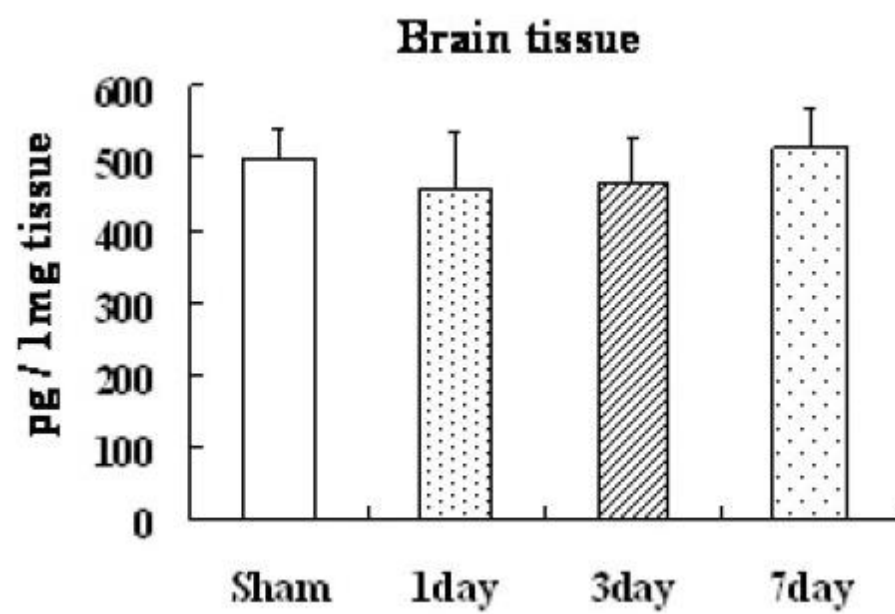


Figure 1.7: Enzyme-linked immunosorbent assay (ELISA) for SDF-1 α in brain tissue.

There is no increase in SDF-1 α protein expression in injured animals compared to sham control animals (Itoh et al., 2009).

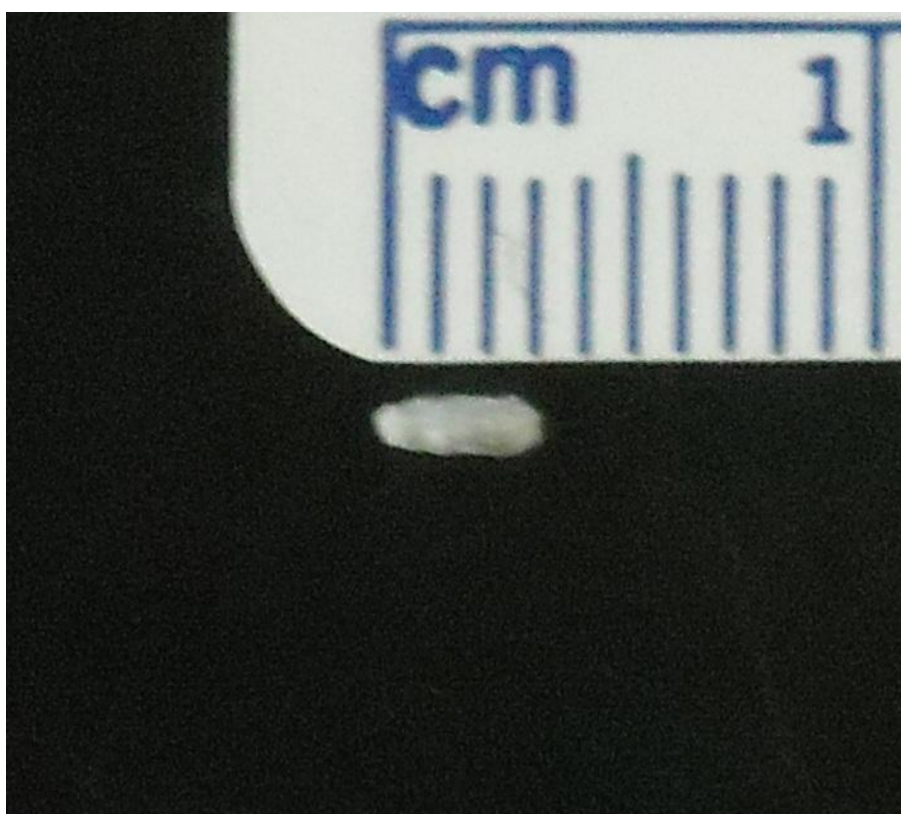


Figure 1.8: Typical alginate thread. A typical alginate thread used in our *in vitro* and *in vivo* investigations. These threads are formed of electrospun fibers and are infused with either saline (as a control) or SDF-1 α .

Chapter 2 – Materials and Methods

2.1 In Vitro Study

Neural Stem/Progenitor Cells

Neural stem cells (NSCs) are self-renewing and multipotent cells which have ability of unlimited division and are capable of differentiating into both neurons and glial cells. Neural progenitor cells (NPCs) have limited capacity for self-renewal and restricted potential for differentiation. While common during neuronal development, NS/NPCs in the adult brain are found primarily in two neurogenic regions, i.e. the subventricular zone and the dentate gyrus of the hippocampus. For *in vitro* experiments in this study, two different sources of NS/NPCs were used. One batch of cells was adult rat hippocampal NS/NPCs purchased from Millipore (Millipore, MA). Another batch of cells was NS/NPCs isolated from the SVZs of adult rats in our lab. Both cell populations were cultured as a monolayer in Poly-L-Ornithine/Laminin coated flasks and maintained under our standard culture conditions with DMEM/F12 supplemented with N2 (1%), bFGF (20ng/ml).

Incorporation of SDF-1 α into Alginate

Alginate is a versatile biopolymer commonly used in studies investigating drug delivery systems. Fabrication of an alginate thread begins with preparation of a stock 10mg/ml solution of alginate in dH₂O. SDF-1 α is then suspended in this solution at a concentration of 1.5 μ g/ml. From the stock solutions, 25 μ L are added to a cylindrical “casting” vessel consisting of a tuberculin syringe. The solution of alginate/chemokine is then placed into the vessel until frozen and then extruded into a 2% calcium bath to induce polymerization. The tube is dried and “cured” in a hexafluoroisopropanol (HFIP) rinse in order to form a filament of alginate for

incorporation into an electrospun scaffold. After scaffolding material has completely covered the alginate thread, the electrospinning process is ended, resulting in a small-diameter alginate thread containing chemokine (Figure 1.8). The scaffold is then cut with a pair of sterile scissors and transferred to an antibiotic culture medium.

Migration Assay Using Boyden Chamber

The Boyden Chamber migration assay consists of a two-compartment system in which a chemoattractant is used to induce migration of cells through a porous membrane from an upper compartment to a lower compartment. The pores of this membrane must be small enough to restrict passive migration while still allowing active migration of cells into the lower compartment (Boyden, 1962). For this study, ThinCert® cell culture inserts were purchased from Greiner Bio-One to examine the effectiveness of SDF-1 α as a chemoattractant for neural stem cells. The experimental setup of the ThinCert® insert is shown in Figure 2.1.

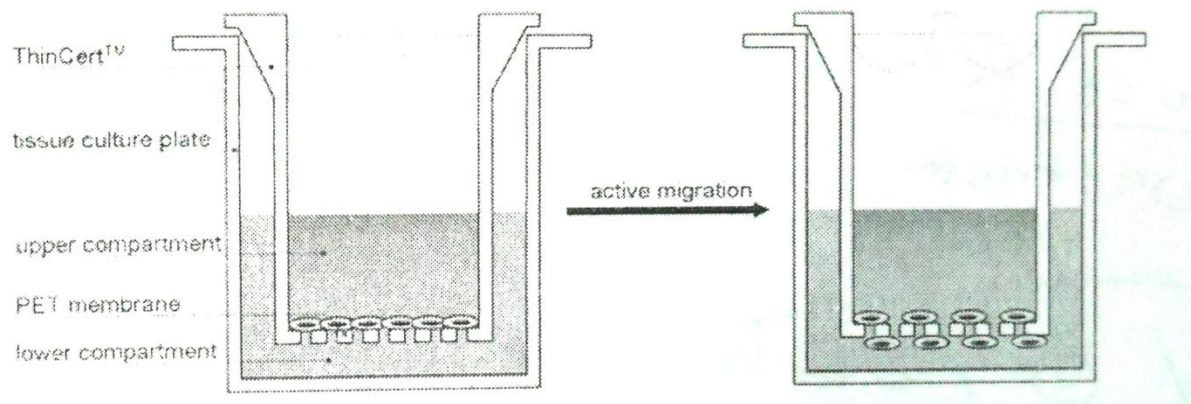


Figure 2.1: Boyden Chamber Migration Assay. The Boyden Chamber setup consists of a ThinCert® insert placed into a well of a typical 24-well plate. As shown, over a period of time active migration will occur, allowing cells formerly plated in the upper compartment to move into the lower compartment, where the chemokine is placed.

For the migration assay, NS/NPCs were cultured as monolayer and maintained in the standard culture condition with DMEM/F12 plus N2 and bFGF until confluent. Cells were then trypsinized, collected and spun down at 1000g for 5 minutes. Cells were resuspended in a serum-free medium to a final concentration of 1.5×10^5 /1.2 ml, depending on number of wells to be used. For Boyden Chamber Assay, 600 μ l of culture medium (with or without chemo-attractant or alginate) was added to each well of the 24-well cell culture plate as shown in Figure 2.2. For each cell culture insert, a total of 200 μ l of the cell suspension (approximately 25,000 cells) was added. The plate was then incubated for 48 hours with SDF-1 α containing medium (through alginate thread or directly added into the medium) in the cell culture incubator. After 48hr incubation, the SDF-1 α containing culture medium was removed from each well of the cell culture plate and replaced with 450 μ l of serum free medium which contains 8 μ M CalCein-AM (Invitrogen). The CalCein-AM solution used contains 3mL DMEM (Invitrogen) with 0.2% BSA (Bovine serum albumin) and 6 μ L CalCein-AM and is used to fluorescently label the cells in the lower compartment. The plate was then incubated for an additional 45 minutes, and the remaining cell culture medium was removed from the cell culture inserts. Thereafter, the cell culture inserts were transferred to a fresh 24-well plate containing 500 μ l of Trypsin/EDTA mixture and then incubated at 37 °C for 5-10 minutes while agitating the plate from time to time to detach all cells from the bottom of the cell culture insert. For measurement of the cell numbers which had migrated out of the culture inserts, the intensity of CalCein-AM fluorescence was measured with a fluorescent plate reader. Briefly, 250 μ l of the solution from each well were transferred to a black flat bottom 96 well plate and the fluorescence of CalCein-AM was read with a plate reader at excitation wavelength 545 nm and emission wavelength of 590 nm.

To test if SDF-1 α can be released after initial 48 hours and the amount released is sufficient to influence cell migration, in the original 24 well plate, extra cells that were plated into 3 cell inserts at the time of initial migration assay and were incubated with culture medium containing fresh SDF-1 α or alginate threads that were taken out of the previous tests. Cell culture, CalCein-AM and migration assay were repeat as described above.

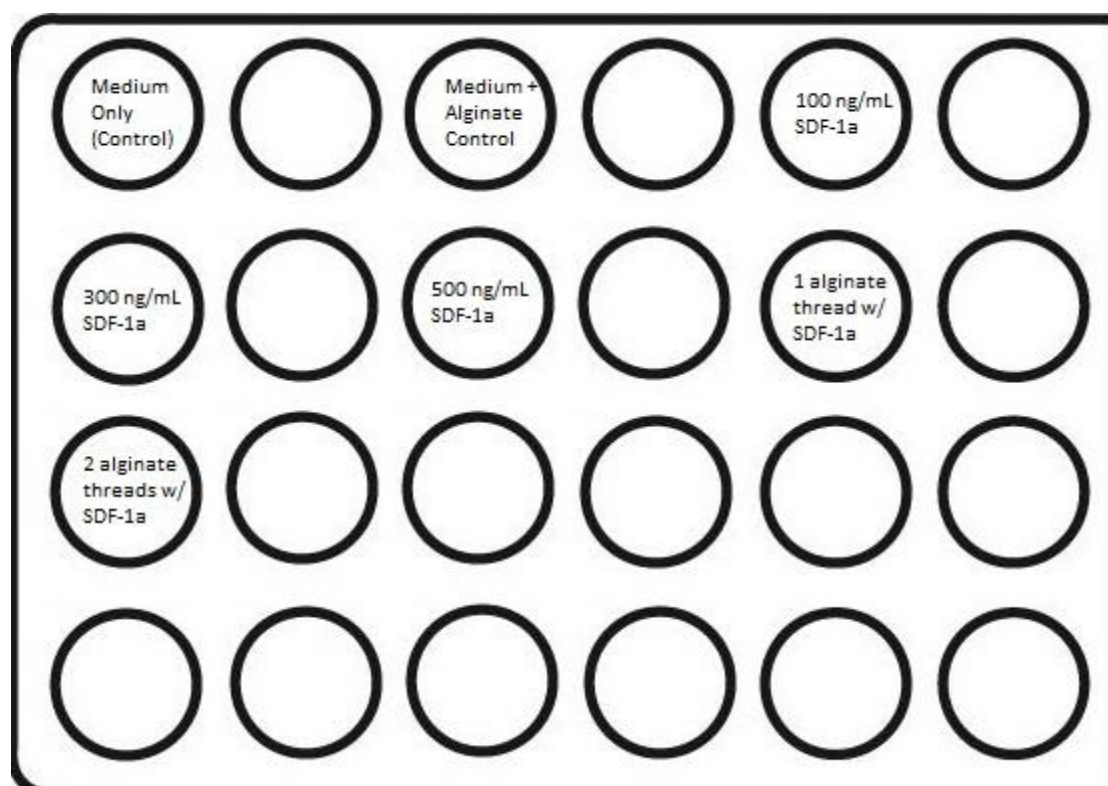


Figure 2.2: Experimental Plate Setup for Boyden Migration Assay. Each of the labeled wells contained one ThinCert® Boyden Chamber insert with plated cells in the upper compartment and the medium in the lower compartment.

Immunocytochemistry for Cultured Cells

To examine the phenotype of the cells at the time of migration assay, extra cells were plated into a coated 24-well plate at the time of plating for Boyden Chamber Assay and cultured with standard culture condition for 48 hr, then washed with PBS and fixed with 2% Paraformaldehyde for 10 min before processed for immunostaining. After wash with PBS, the cells were then blocked with 10% normal horse serum (NHS) in PBS for 30 minutes followed by incubation at room temperature (RT) for 1 hr with the following primary antibodies: stem cell markers GFAP (1:1000, Dako), Nestin (1:500, Chemicon) and SOX2 (1:500, Millipore), immature neural marker Tuj1 (1:1000, Covance), and oligodendrocyte progenitor cell marker NG2 (1:500, Millipore). After incubation, cells were washed with PBS and then incubated with Alexa fluor 488 anti-mouse IgG or Alexa Fluor 568 anti-rabbit IgG at 1:200 dilutions at RT for 30 minutes. The cells were then washed with PBS before incubating with DAPI (1:1000, Invitrogen) for 10 minutes to label cell nuclei. Finally, cells were washed with PBS and quick rinsed with dH₂O before being mounted with flouromount.

2.2 In Vivo Study

Animal Subjects

All animal protocols used for this study followed guidelines that were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University. These procedures followed guidelines established in the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services). A total of 34 male Sprague-Dawley rats (Harlan) aged at 3 months weighing approximately 300g were used in the study. These animals were housed with two in each cage with food and water available *ad*

libitum. The animal room where these rats were kept was maintained at a temperature of approximately 20°C with a 12 hour light/dark cycle.

Surgical Preparation and Procedure

Animals were subjected to a moderate controlled cortical impact (CCI) injury following a standard protocol (Dixon et al., 1991). All surgical tools were sterilized and aseptic surgical procedures were followed throughout the surgical processes. Each rat was anesthetized in a Plexiglas chamber with 5% isoflourane, the head was shaved and the animal was secured in a stereotaxic apparatus. Throughout surgery, the animal was ventilated via nose cone with 2.5% isoflurane in a gas mixture (30% O₂, 70% N₂). The shaved region of the head was scrubbed with Betadine to disinfect the area, and Puralube ointment was applied to the eyes to prevent dryness. After a midline incision was made to expose the skull, the underlying connective tissue was retracted using hemostats. A 4mm craniotomy was made on the left parietal bone slightly behind the bregma suture using a trephine and a Dremel drill fitted with a small dental drill bit (Figure 2.3). After sufficient drilling, the bone chip covering the brain was removed and the edges of the craniotomy were carefully cleaned to ensure no bone fragments remained.

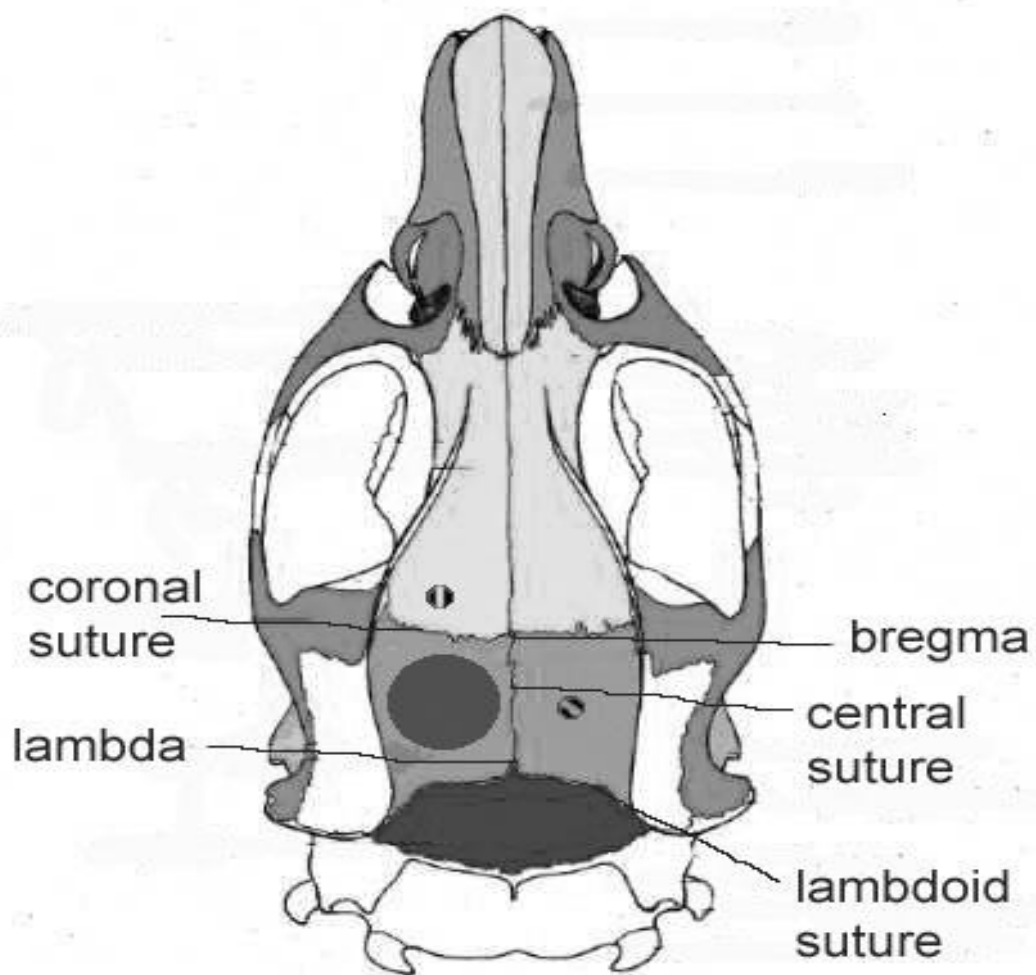


Figure 2.3: Craniotomy and injury site. A 4 mm craniotomy was made in the left parietal bone slightly behind the bregma suture. This served as the site where the CCI injury was delivered.

Controlled Cortical Impact Injury (CCI)

Controlled Cortical Impact Injury (CCI) model was used for this study because it has been shown to produce a reproducible focal traumatic brain injury (Dixon et al., 1991). All injured animals were prepared with the standard surgical protocol according to IACUC and DAR regulations. The CCI injury device (Figure 2.4) was carefully adjusted and the impactor tip was perpendicular to the brain surface, so as to not pierce the dura surface during injury. A 3mm steel tip was used. Once the craniotomy was made, the anesthetic was switched off. After the animal regained tail reflex, a mild to moderate injury was produced with the velocity of 4.0 m/s with a dwell time of 0.05 sec. After injury, the scalp was sutured and the animals were allowed to recover. The time of animal to regain toe and tail reflexes as well as the righting time was recorded to assess the severity of injury. The animals were observed until normal breathing rhythm had returned. For control, sham animals went through the same surgical procedure without injury impact.



Figure 2.4: Photograph of a typical CCI injury device. The actual pneumatic injury mechanism is shown on the right of the photograph. The control panel to the left of the photograph is used to control the velocity and dwell time of the impactor.

Introduction of SDF-1 α to the Cerebral Cortex

To test if SDF-1 α can induce cell migration in normal brain, SDF-1 α was stereotactically injected into the cerebral cortex close to the rostral migratory stream. For injection, animals were anesthetized with 5% isofluorane and secured on a stereotaxic frame ventilated with 2% isofluorane. A midline incision was made and a burr hole was made with a dental drill at the following coordinates: AP +0.5mm, ML +2.0mm, DV -3mm. A 10 μ l Hamilton syringe with a 26G beveled needle was mounted on an automated syringe driver to deliver 3 μ l of SDF-1 α (200ng/ μ l) for each injection site at a rate of 0.5 μ l/min. To prevent back flow along the needle track, the needle was left *in situ* for 5 min prior to and after cell injection. Control animals received PBS injection only. The target area for the injection was an area slightly above the rostral migratory stream. This is to ensure that the injected SDF-1 α is at close enough range to influence the migration of the neuroblasts originated from the SVZ. The injection site also had to be far enough away from the RMS so that we could clearly discern that the migrating cells had left the migratory stream and was moving toward the SDF-1 α injection site. Equal volume of sterile PBS was injected into the contralateral site of the cortex as saline control. After injection, the incision was sutured and animal was returned to a clean warm cage before back to the animal facility.

Insertion of Alginate Threads for SDF-1 α Delivery

In CCI injured animals, SDF-1 α was administered through the use of alginate threads. At 2 days after CCI injury, injured rat was anesthetized with 5% isoflourane and secured on a stereotaxic frame ventilated with 2% isoflourane. The incision site from the CCI surgery was reopened, and the surgical site was extensively cleaned. A single alginate thread containing

SDF-1 α or two pieces of alginate thread cut from one thread were gently inserted into the injured cortex. Bone wax was used to cover the craniotomy site before the incision was sutured. The animal was placed into a warm cage for recovery before returning to the animal facility.

Tissue Processing

Animals were sacrificed at either 7 or 14 days post-injury (d.p.i.) with overdose isoflourane anesthesia, then transcardially perfused with 200 ml 1X phosphate buffered saline (PBS) followed by 200 ml 4% paraformaldehyde (PF) in PBS. Immediately after perfusion the brains were removed and further fixed in 4% paraformaldehyde. After at least 48 hours fixation, the brains were removed from PF and sliced coronally at 60 μ m thick using a vibratome. Sections were collected between bregma +1.70mm and -3.30mm spanning through the lateral ventricle and the rostral portion of the hippocampus. Sequential sections were collected into four 24-well plates and stored in a solution of PBS and 0.01% sodium azide at 4°C before processed for immunostaining.

Immunohistochemistry

From each of the sacrificed animals, a full tray of 24 brain slices was processed for doublecortin (DCX) staining. DCX is a microtubule-associated protein expressed by migratory neuroblast cells (Brandt et al., 2003).

DCX Immunostaining

Sequential coronal sections stored in PBS + 0.01% sodium azide at 4 °C were first allowed to return to room temperature, then washed with PBS 2 times for 5 minutes. Endogenous peroxides were quenched with 3% H₂O₂ at room temperature for 1 hour. Sections were then blocked

overnight in blocking buffer (5% normal horse serum in 1X PBS + 0.3% Triton X100) and followed by incubation with goat anti-DCX IgG (Santa Cruz) diluted at 1:100 concentration in the same blocking buffer used before. After 48 hours of incubation in the primary antibody, sections were washed with 1X PBS with 0.3% Triton X100 3 times for 10 minutes per wash. The secondary antibody, biotinylated anti-goat IgG (Jackson Laboratories, ME), was also prepared in blocking buffer at 1:200 dilution and added to each well. The sections were incubated for 24 hours at 4°C. After washing with 1X PBS + 0.3% Triton X100 3 times for 10 minutes, avidin-biotin complex (ABC kit, Vector Laboratories) prepared 30 minutes before use at a concentration of 1:200 avidin and 1:200 biotin diluted into 1X PBS was added to each well. The sections were incubated at room temperature for 2 hours and then washed in 1X PBS 3 times for 10 minutes. A solution of 5, 5-diaminobenzidine (DAB) was then prepared by adding 100 mg of powdered DAB to 40 ml of 0.1 M phosphate buffer and 17.5 μ L of 30% hydrogen peroxide. After addition of the DAB solution, sections were observed under a dissecting microscope until the staining was adequate. The DAB solution was then quickly removed and the sections were washed with 1X PBS 2 times for 5 minutes and stored at 4 °C before mounting onto glass microscope slides.

CXCR4 Immunostaining

To label cells that express SDF-1 α receptor CXCR4, selected sections through the SDF-1 α injection site or through injury cavity were processed for CXCR4 staining. The staining procedure was similar to DCX immunostaining. The primary and secondary antibodies used were rabbit anti-CXCR4 primary antibody (1:500, Santa Cruz) and biotinylated anti-rabbit IgG secondary antibody (1:200, Vector Laboratories).

Cresyl Violet Counterstaining

After mounting on glass microscope slides, sections were allowed to dry overnight. For counterstaining, slides were hydrated in distilled water for 2 minutes and then stained with 0.1% cresyl violet solution for approximately 1 minute. The sections were then washed in running tap water and then sequentially placed into increasing concentrations of ethanol, beginning with 70%. The mounted slides were taken through a progression of another 70% and one 90% ethanol, followed by differentiation of the stain in acidic alcohol (90% ethanol with a few drops of acetic acid). Sections were then dehydrated in 100% ethanol twice for 1 minute each, followed by placement in Citrisolv (Fisher Scientific) twice for 5 minutes each. The slides were then cover slipped using Permount and allowed to dry.

Stereological Quantification

Quantification of the number of DCX-positive cells was carried out in the cortical gray matter and portions of the upper white matter tract above the rostral migratory stream. To quantify the number of migratory neuroblasts, DCX-stained sections were examined with an Olympus Image System CAST Program (Olympus, Demark). The specific region of interest in the ipsilateral side was outlined under a 4x objective, and DCX-positive cells were then counted using the 20x lens, focusing through the thickness of the section. All DCX-positive cells located in the region of interest were counted. The dissector height (h) was known relative to the section thickness (t). With these parameters, the number of total cell counts (N) was estimated as $N = \Sigma Q^{-} \cdot (t/h)(4)$, where ΣQ^{-} was the number of cells counted.

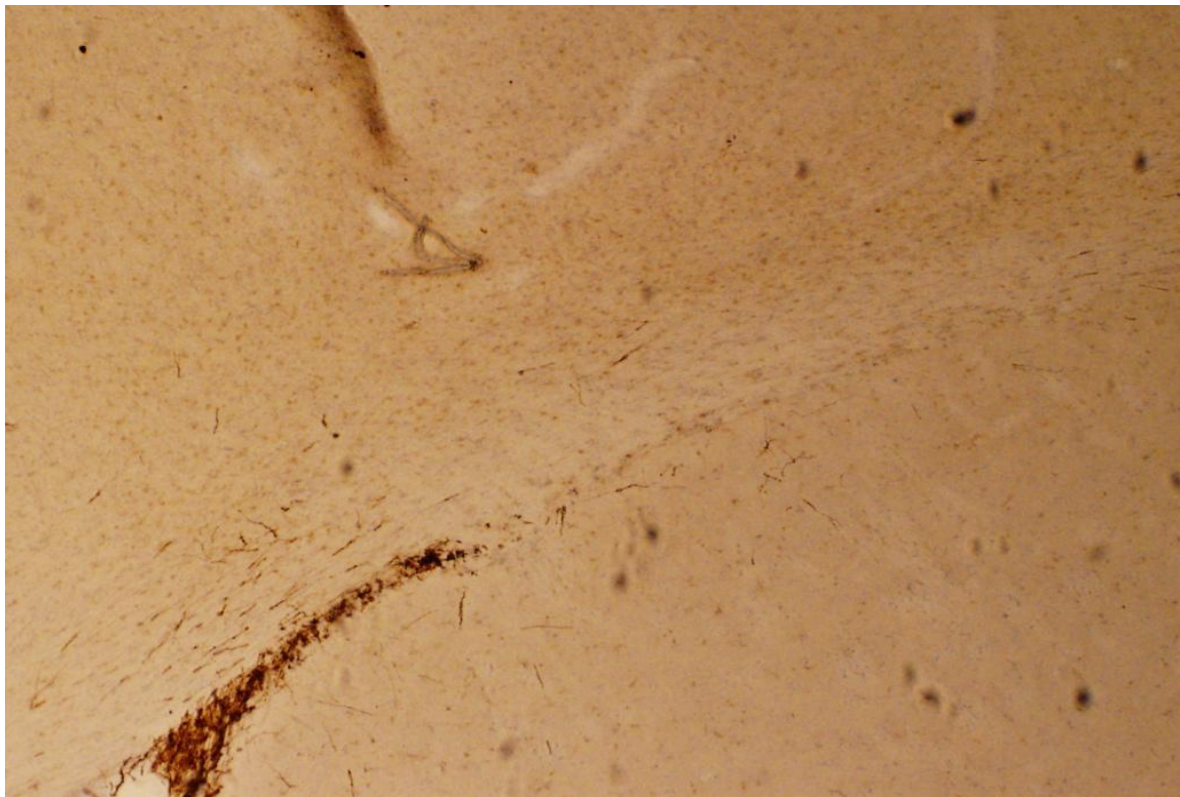


Figure 2.5: Image of the DCX-stained rat brain section. This image shows the upper regions of the SVZ, the rostral migratory stream (RMS), and parts of the white matter tract of an animal that has been subject to a moderate CCI injury. The injury cavity resulting from this injury would be slightly above the frame of this photograph. Migrating DCX-positive cells are shown as darkly stained brown cells in the SVZ and the white matter tract.

Chapter 3 – Results

Based on the findings of previous research studies, traumatic brain injury (TBI) results in increased neurogenesis within the subventricular zone and the chemokine SDF-1 α can induce cell migration. In the present study, the goal is to determine if introduction of SDF-1 α to injured cortical tissue can enhance migration of the newly generated neurons towards the site of injury after TBI. The ability of SDF-1 α to induce cell migration was tested both *in vitro* with cultured neural stem cells and *in vivo* in CCI injured animals. SDF-1 α was delivered either directly or through alginate threads for slow delivery. For *in vitro* study the effects of inducing cultured cell migration were assessed with a Boyden chamber migration assay. For the *in vivo* study, a total of 34 Sprague-Dawley rats were utilized. After an allotted period of time, the animals were sacrificed and the brains were removed. DCX, which labels migrating neurons, was used to analyze the effects of SDF-1 α in inducing cell migration *in vivo*.

3.1 *In Vitro Study*

3.1.1 *Phenotypes of cultured cells used for migration assay*

For the migration assay, we used two different sources of NS/NPCs. NS/NPCs are capable of generating both glial cells and neurons, and cell differentiation normally occurs with increasing passage. As the migration speed of neurons and glial cell towards a chemokine may be different, it is necessary to identify the cell types at the time when migration was assessed. To do this, we plated extra cells from the same passage used for the Boyden Chamber into a separate 24-well plate and fixed the cells at the time of migration assay. Several markers for NS/NPCs were used including GFAP (an intermediate filament protein expressed by NS/NPCs and mature astrocytes), nestin (an intermediate filament protein expressed by NS/NPCs and activated mature

astrocytes), and Sox2 (a transcription factor expressed by NSCs). To determine the possible lineage of these cells, NG2 (marker for oligodendrocytes precursor) and immature neuronal marker Tuj1 were also used. DAPI was used for nuclei staining. We found that the majority of cells used in the lower passage for both cell sources were nestin-positive cells (Fig. 3.1A), and cells used in the higher passage have lower GFAP/nestin expression (Fig. 3.1C, D) and high NG2 expression (Fig. 3.1B). This suggests a phenotypical change of cultured cells with time.

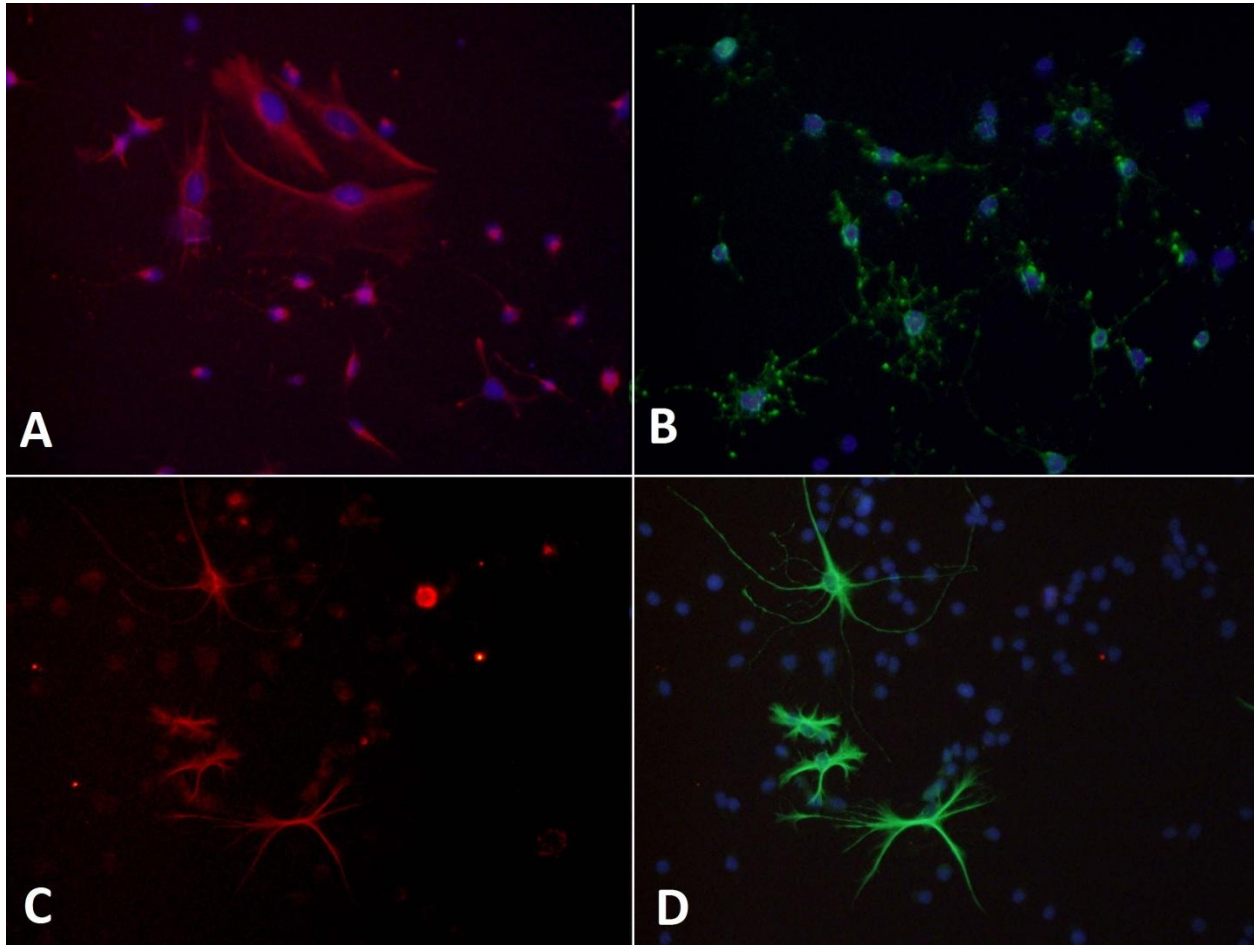


Figure 3.1: Cell types of cultured NS/NPCs. **A)** At lower passage (2-4), the majority of cultured cells stained positive for nestin (red). **B)** Many cells in the higher passage express NG2. Nuclei are identified by DAPI (blue) staining. **C and D)** At higher passage (5-7), the percentage of GFAP (green)/Nestin (red)-positive cells is reduced.

3.1.2 SDF-1 α induces dose dependent migration of cultured NS/NPCs

The Boyden Chamber migration assay is used to investigate the ability of soluble chemokines such as SDF-1 α to influence migration of cells across a specified distance. To measure the number of cells that have migrated towards SDF-1 α containing lower chamber, cells were labeled with CalCein-AM and then trypsinized for assay. The fluorescence of internalized CalCein-AM was then measured with a microplate reader to quantify the number of cells that have migrated (Figure 3.2).

We first assessed the effect of SDF-1 α induced cell migration after 48 hours exposure. We found minimal levels of cell migration in the two control wells (medium only and alginate with PBS). In wells with SDF-1 α added directly in medium, 100ng of SDF-1 α exhibited a minimal effect in inducing cell migration whereas 300ng and 500ng of SDF-1 α induced a dose-dependent increase in cell migration, with 500ng of SDF-1 α inducing the maximal effect of cell migration (Fig.3.2). SDF-1 α incorporated into alginate threads also induced cell migration and a similar dose-dependent response was observed, with one thread inducing a migratory response of close to 2000 cells compared to two threads showing over 6000 NSCs migrate across the membrane (Fig.3.2). The level of cell migration induced by SDF-1 α /alginate thread was proportionally lower compared to what was found in 300ng and 500ng of SDF-1 α in medium, indicating that 1 thread released less than 300ng of SDF-1 α whereas 2 threads released less than 500ng of SDF-1 α during the first 48 hours.

We next examined if a SDF-1 α -containing alginate thread can continually release sufficient SDF-1 α to induce cell migration after the first 48 hours. To do this, we reused the threads from the first 48hr assay. Cells in the chamber inserts were cultured with the alginate threads for another 48 hr, and CalCein-AM fluorescence was measured. We found that SDF-1 α release from the alginate threads at days 3-4 was sufficient to induce cell migration and produced a similar dose-dependent effect compared to the first 48-hour assay (Fig.3.3).

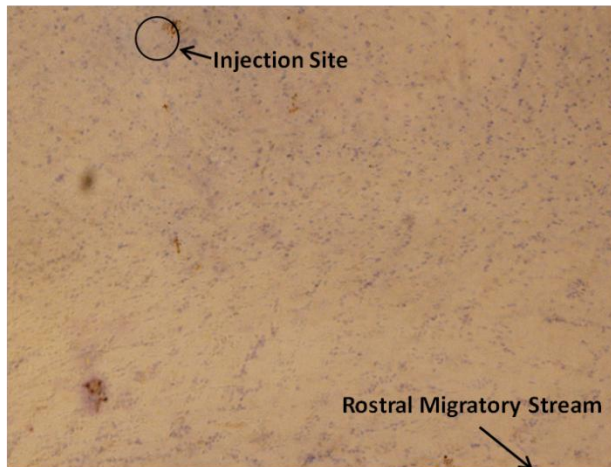
Figure 3.2: Boyden Chamber migration assay (first 48 hours). The graph shows the number of cells migrating from the upper compartment into the lower compartment during 48 hr incubation. Column 1 shows the response with a lower compartment filled with media only. In column 2, a single control alginate thread filled with saline was added to the medium. For columns 3, 4, and 5, varying concentrations of SDF-1 α were added in the media of the lower chamber. Columns 6 and 7 both contained medium with one SDF-1 α containing alginate thread to column 6 and two SDF-1 α /alginate threads to column 7. Note a dose-dependent increase of cell migration when SDF-1 α was added in the medium or in the alginate thread.

Figure 3.3: Boyden Chamber migration assay (second 48 hours): The graph shows the effect of SDF-1 α in alginate thread in inducing cell migration during the second 48 hour period (days 3-4). The control well with medium only is shown in column 1. Column 2 shows the migratory response to a single alginate thread which was used in column 6 of Figure 3.5. Column 3 shows migration response in two alginate threads used in column 7 of Figure 3.5. Note significant cell migration was induced in both 1 and 2 threads wells compared to the medium only well. Two threads also produced higher migration than that of one thread.

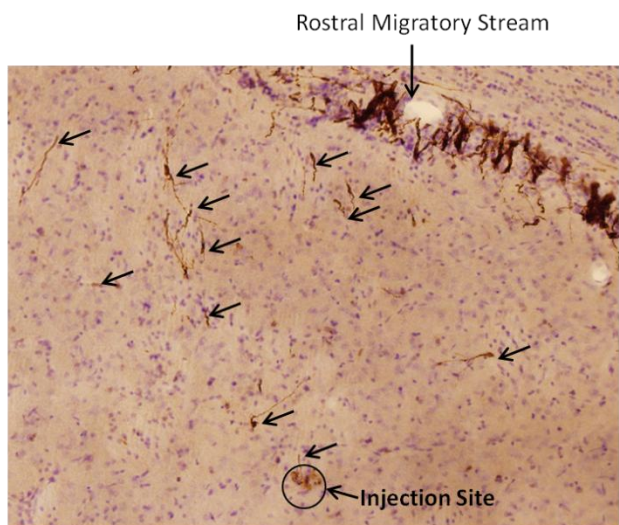
3.2 In Vivo study: Effects of SDF-1 α on newly generated neuroblasts migration following TBI

3.2.1 Intracerebral injection of SDF-1 α inducing limited cell migration

To test whether SDF-1 α can induce migration of newly generated neurons derived from the SVZ *in vivo*, we first injected SDF-1 α directly into the cerebral cortex in an area close to the rostral migratory stream where these migrating neurons travel in normal situations. DCX was used to label the migrating neurons. In the control cortex where 3 μ l of sterile PBS was injected, there were very few DCX-labeled cells along the injection tract and deposition site (Figure 3.4). In the cortex where a single 3 μ l SDF-1 α dose at the concentration of either 100 ng/ml or 200 ng/ml was injected, a larger population of DCX-labeled cells was found at the injection site (Fig.3.4). Quantification of the number of DCX-positive cells present around the site of injection using stereological method revealed that there were a significantly higher number of DCX-positive cells in the SDF-1 α injected cortex compared to the saline control side (Figure 3.5). This data suggests that a single injection of SDF-1 α can attract migrating neuroblasts away from their normal route towards the injection site in some degree.



Uninjured Brain, Injected with single dose of saline



Uninjured Brain, Injected with single dose of SDF-1a (injection site)

Figure 3.4: Injection of SDF-1 α induced cell migration. Naïve rats were given 3 μ L injections of either saline or SDF-1 α at the site right above the RMS. DCX-stained coronal sections were taken from an animal injected with saline (left) or SDF-1 α (right) and sacrificed 7 days later. Saline injection has a minimal effect of inducing cell migration whereas single injection of SDF-1 α induces slightly more migrating neuroblasts to move towards the injection site.

Figure 3.5: The number of DCX-positive cells in the uninjured brain following SDF-1 α or saline injection. In control animals injected with saline only, minimal levels of NSC migration were seen. Conversely, subjects injected with SDF-1 α saw increased levels of DCX-positive cells (p=0.30).

3.2.2 Neuroblast Migration following CCI Injury and following Administration of SDF-1 α through Alginate Treads

Previous studies have found that focal ischemic injury can enhance cell proliferation in the SVZ and induce neuroblast chain migration towards the infarction site, possibly under the chemoattractive action of SDF-1 α released by the ischemic tissue (Imitola et al., 2004, Parent et al., 2002). Studies have also found that focal brain injury induced by CCI injury can enhance cell proliferation in the SVZ (Dash et al., 2001). However, it was not clear whether focal brain injury can also induce neuronal migration towards the injured site and potentially replace the damaged neurons. In the current study, we examined whether focal brain damage induced by CCI injury can attract neuroblast migration from the SVZ to the site of injury using DCX staining. We also examined whether administration of exogenous SDF-1 α through alginate thread to the focal injury site can promote this migration.

CCI injury was produced in adult male Sprague-Dawley rats. A 2.2 mm impact was administered to one side of the cerebral cortex region. Experimentation in our lab has shown this injury level produces a mild to moderate injury with a focal cavity formation limited to the cerebral gray matter of layers I-V (Figure 3.6). Two days following injury, control animals received no further treatment (n=5) and experimental groups received either 1 piece of SDF-1 α -containing thread or two pieces of thread cut from a single thread (n=5). Animals were left to survive for 1 or 2 weeks after the implantation. DCX was used as a marker for neurogenesis and migrating neurons. In the CCI injured animals without SDF-1 α treatment, many DCX-positive cells were found in the ipsilateral side of the SVZ and the dentate gyrus, confirming that CCI injury enhanced neurogenesis in the neurogenic regions compared to the contralateral side. More

DCX-positive cells were also observed along the RMS in the injury ipsilateral side (Figure 3.7). A small number of DCX-positive cells in the ipsilateral cortical gray matter underneath the injury cavity were also observed (Fig. 3.7). In injured animals which received implantation of SDF-1 α /alginate thread, there were more DCX-positive cells along the ipsilateral RMS and in the cortical gray matter close to the injury cavity (Figure 3.8). Stereological quantification analysis showed that the number of DCX-positive cells at the ipsilateral cortical region close to the injury site was significantly higher in the SDF-1 α /alginate treated animals compared to the untreated group (Figure 3.9). We did not find a difference in animals sacrificed at 7 or 14 days after SDF-1 α /alginate thread implantation nor thread in one piece or cut into two pieces, so the data was pooled together.

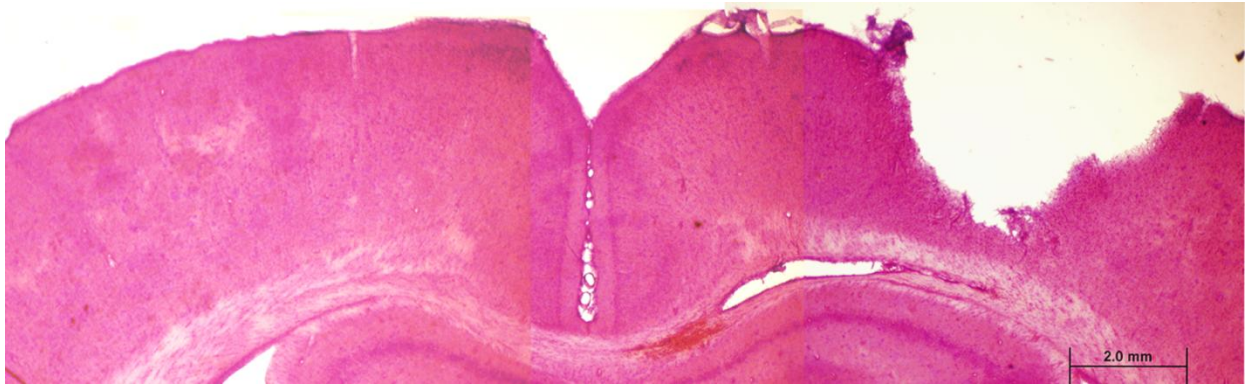
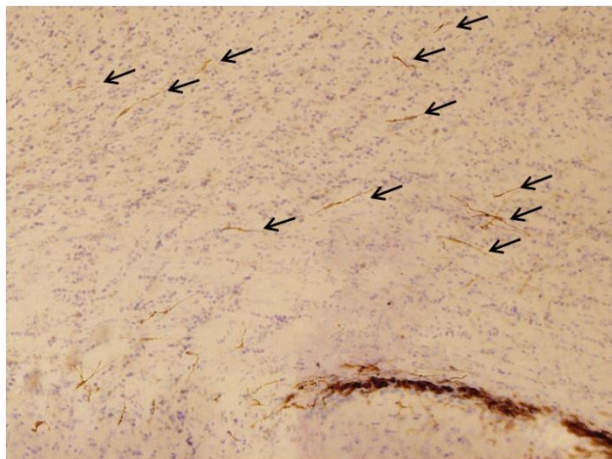
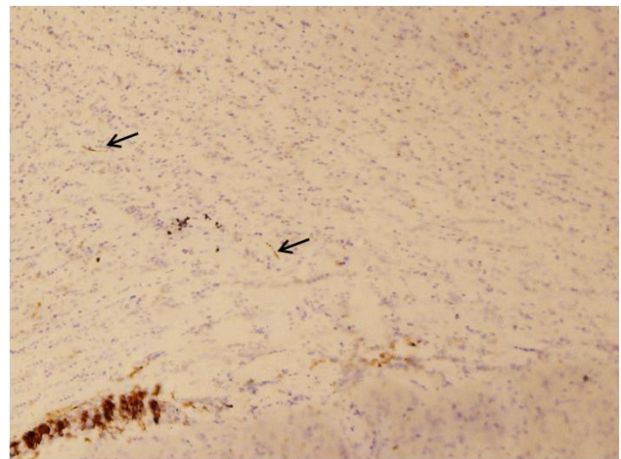


Figure 3.6: Coronal section through the injury site of a rat brain following CCI injury. A moderate CCI injury results in the formation of a medium-sized injury cavity in the cortex, similar to what would be seen in a human subject receiving a similar focal injury.



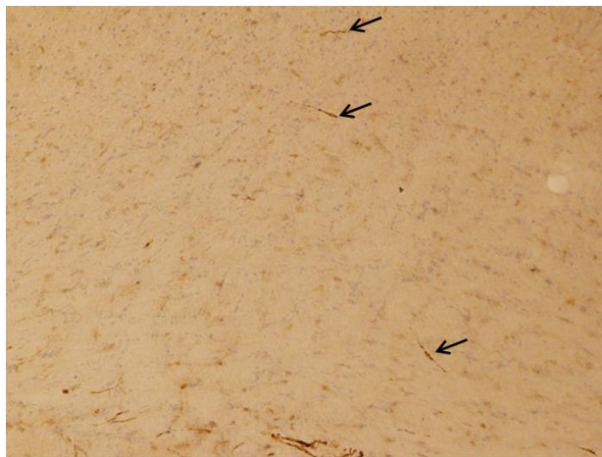
Injured Brain, No treatment



Uninjured Brain, No treatment

Figure 3.7: DCX-labeled migrating neurons in the ipsilateral cortex following CCI injury.

DCX-stained coronal sections taken from an uninjured animal (right) and the ipsilateral side of cortex of a CCI injured animal at 7 days post injury (left). The SVZ of the lateral ventricle is located slightly out of the image below the rostral migratory stream. The picture on the left shows DCX-positive cells (arrows) migrated out of the white matter tract and into the cortical gray matter adjacent to the injury site (the injury cavity is not pictured at this resolution and is located slightly above the upper extent of this frame). The picture on the right shows same regions from an uninjured brain, where very few DCX-positive cells are found.



Injured Brain, No Treatment



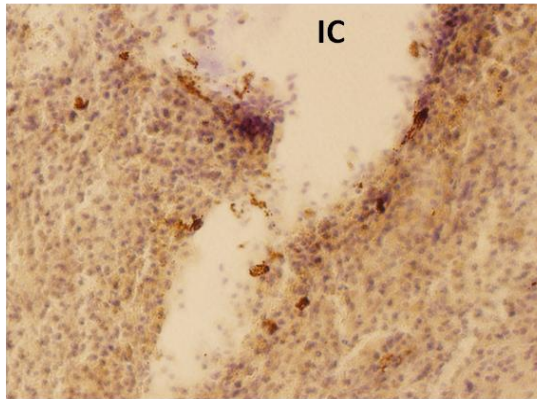
Injured Brain, Treated with SDF-1a Thread

Figure 3.8: Migration of DCX-positive cells in injured brains following SDF-1 α /alginate thread implantation. DCX-stained coronal sections taken from a CCI animal without SDF-1 α treatment (left) and an animal which received SDF-1 α /alginate thread implantation (right) and sacrificed at 7 days later. Note many more DCX-labeled migrating neurons (arrows) along the ipsilateral RMS and at the cortical area adjacent to the injury in the animal receiving an alginate thread.

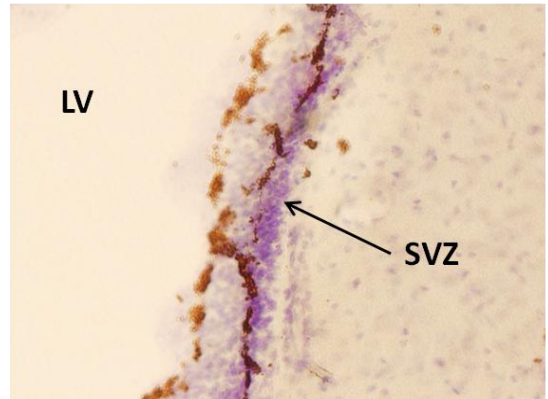
Figure 3.9: The number of DCX-positive cells in the cortical region near the injury cavity following CCI with or without SDF-1 α . Stereological quantification of DCX-positive cells in the injured rat brains showed increased number of cells in animals treated with SDF-1 α compared to CCI-only animals (p=0.10).

3.2.3. Expression of SDF-1 α receptor CXCR4 in the SVZ and cortical tissue

As reported before, the only known receptor for the chemokine SDF-1 α (CXCL12) is CXCR4, a membrane bound receptor that is heavily involved in cell proliferation and migration (Imitola et al., 2004). To examine the expression profile of SDF-1 α and its receptor CXCR4 in the adult brain, we stained brain sections taken from animals that received injections of SDF-1 α or CCI injury with or without alginate thread implantation. The antibody against SDF-1 α did not show staining in any sections, possibly due to the specificity of the antibody. The antibody against CXCR4 stained cells along the SVZ and some cells with macrophage/microglia morphology around the injury cavity and along the ipsilateral corpus callosum (Figure 3.10) in sections taken from CCI injured animals.



CXCR4 Expression at Injury Site



CXCR4 Expression at SVZ

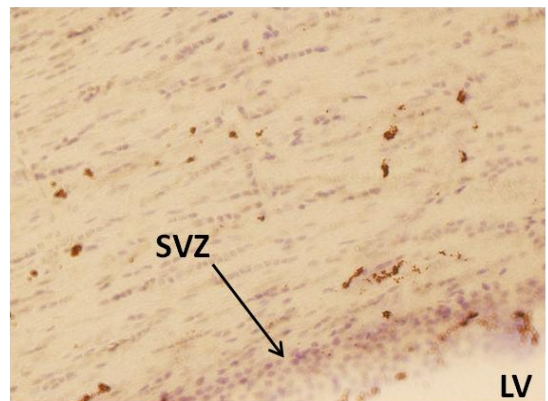
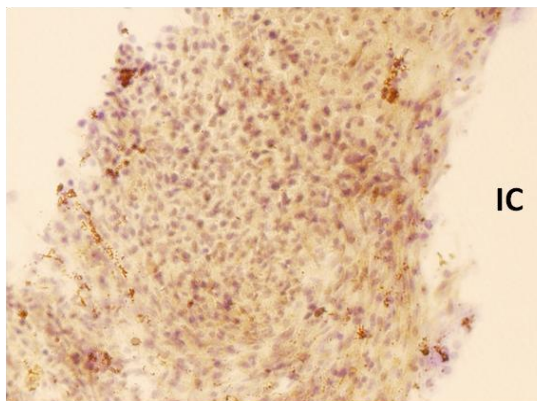


Figure 3.10: CXCR4 expression in the brain following TBI. Photographs taken from the CCI injured brain at 7 days post injury. CXCR4-positive cells as shown in dark brown color were found along the SVZ in the wall of the lateral ventricle (LV) and around the injury cavity (IC).

Chapter 4: Discussion

Traumatic brain injury (TBI) is one of the most important healthcare concerns in the United States for which adequate treatment has yet to be discovered (Langlois et al., 2006). The characteristics of TBI include extensive tissue damage due to primary mechanical insults as well as secondary cellular insults which result in continued neural damage (McIntosh et al., 1996). Researchers in the field of TBI have established that the Central Nervous System (CNS) has a limited capacity for regeneration and repair following injury (Altman and Das, 1965; Lois and Alvarez-Buylla, 1993). Neural stem/neural progenitor cells (NS/NPCs) persist in the adult CNS throughout life and provide a source for new cells that could improve a patient's chances for functional recovery. These neurogenic cells are found in two regions of the adult brain where they constantly produce new neurons during normal brain function: the subventricular zone (SVZ) of the lateral ventricle and the dentate gyrus (DG) of the hippocampus (Sun et al., 2005; Chirumamilla et al., 2002; Temple and Alvarez-Buylla, 1999). Related research also concluded that TBI increases division and proliferation of these neural stem cells. These discoveries are all positive findings; however, further analysis has found that the regenerative capacity of the cells is limited and only results in minimal levels of tissue regeneration after injury (Kuhn et al., 1996).

In an adult brain under normal conditions, newly generated NS/NPCs in the SVZ migrate along the rostral migratory stream (RMS) and cells in the subgranular zone (SGZ) migrate laterally into the granule cell layer of the DG (Doetsch et al., 1997; Garcia-Verdugo et al., 1998). Movement of these neuroblasts is controlled by a number of secreted molecules and guidance cues, including the chemoattractant SDF-1 α (Hagg et al., 2005). Following TBI or stroke, migration of newly generated neuroblasts to the site of injury occurs and this migration is linked

to the focal release of chemokine SDF-1 α and the upregulation of its receptor CXCR4 expression at the site of ischemic injury (Robin et al., 2006; Kojima et al., 2010). As SDF-1 α /CXCR4 plays an important part in mediating cell migration, we attempted to utilize this pathway to attract and guide TBI-enhanced newly generated neuroblasts to the site of injury. This SDF-1 α /CXCR4 interaction was examined in both *in vitro* and *in vivo* environments using various methods of delivery.

Results Summary

The present study consisted of two distinct experiments in order to determine the chemotactic properties of SDF-1 α . For optimal delivery, we incorporated SDF-1 α into alginate threads. Next we examined the bioactivity of SDF-1 α released from the alginate thread by its action in inducing migration of adult derived NS/NPCs using the Boyden chamber migration assay. We found that the chemoattractant SDF-1 α released from the alginate thread was able to influence cell migration in a dose-dependent manner. In the *in vivo* part of this project, we first assessed the ability of intracerebral injection of SDF-1 α to induce neuroblast migration. Compared to saline injection, a single bolus injection of SDF-1 α into the cortical area near the RMS can induce a limited degree of cell migration towards the injection site. Following this, we tested the effects of SDF-1 α released from the alginate thread in inducing neuroblast migration in the injured brain. In this study, SDF-1 α -containing alginate threads were placed in the injured cortical tissue at 2 days following a focal CCI injury. Increased numbers of DCX labeled migrating new neurons had migrated off the normal RMS towards the injury site. Collectively, the data obtained from the current study suggests: 1) alginate can be utilized for slow release of chemokine SDF-1 α ; 2) SDF-1 α released from alginate is bioactive and can not only induce cell migration *in vitro* but can also act as a guidance cue for cell migration *in vivo*; 3) *In vivo* delivery of SDF-1 α through alginate could be utilized as a therapeutic strategy for endogenous neural repair and replacement after TBI.

SDF-1 α has chemotactic properties and is able to induce NS/NPC migration in vitro

SDF-1 α is a known chemoattractant agent inducing cell migration (Ohtani et al., 1998; Stumm et al., 2002; Tran and Miller, 2003). Published studies have shown its effect on cell migration in different types of cells including NS/NPCs (Bagri et al., 2002; Ponomaryov et al., 2000). In our study, we observed that SDF-1 α can induce cell migration in adult derived rat NS/NPCs, either at the stem cell stage or at more differentiated progenitor stages when SDF-1 α is added directly into the culture medium or through alginate. We have also found that SDF-1 α induced cell migration is dose-dependent. When 100ng of SDF-1 α was added into the medium, very low levels of cell migration similar to control was observed. At 300ng or 500ng SDF-1 α , increased levels of cell migration were observed and were dose-dependent, indicating SDF-1 α has strong chemotactic properties and is able to induce migration of neural stem cells *in vitro*. These results support previous findings showing the effectiveness of SDF-1 α as a chemotactic molecule in inducing migration of NS/NPCs (Imitola et al., 2004).

Use of alginate for SDF-1 α introduction allows sustained chemokine release over a longer period of time

Alginate has been used for delivery of varying types of agents such as growth factors, cytokines, and other drugs. The advantages of alginate delivery include biodegradation, low levels of inflammation, and sustained release of chemicals into a focused region over a period of several days (Tonnesen and Karlsen, 2002). Taking advantage of this delivery method, we incorporated SDF-1 α into alginate threads and tested the release kinetics and bioactivity of SDF-1 α . This migration assay was utilized to test whether threads of alginate containing SDF-1 α would be able to influence migration of cultured adult derived NS/NPCs. Addition of a single

SDF-1 α containing thread to medium resulted in cell migration at levels comparable to levels when 300ng SDF-1 α was added directly to the medium. The single thread contains a total concentration of SDF-1 α much higher than 300ng, but likely releases only a small percentage during the first 48 hour period. When two threads of alginate were added into the medium, higher levels of cell migration were observed, suggesting that high doses of SDF-1 α were released through two alginate threads.

Upon conclusion of the original 48 hour migration assay, we tested the ability of alginate threads to provide a sustained release of chemokine over a longer time period. Using the same alginate threads from the first 48 hours, we found that the SDF-1 α can be further released to induce meaningful cell migration. Since previous studies have suggested that constant exposure to SDF-1 α is required for migration to result, sustained release over a period of days to weeks is needed for continual migration. These results confirm our hypothesis that alginate threads infused with SDF-1 α would be able to release the chemoattractant over a period of several days at a sufficient concentration to induce cell migration.

Alginate has a high capacity for hydration, allowing large amounts of SDF-1 α to be added to the threads and be released over an extended period of time (Tonnesen and Karlsen 2002). Thus, introduction of an alginate thread hydrated with SDF-1 α into the injury cavity of a TBI victim may allow sustained introduction of SDF-1 α to injured cortical tissue.

Injection of SDF-1 α into uninjured tissue results in NSC migration to the injection site

Previous studies have shown the SDF-1 α /CXCR4 pathway mediating tumor migration during tumor metastases (Lau et al., 2010) and neuroblast migration to the ischemic injury site

(Parent et al., 2006). Studies have also found that introduction of SDF-1 α into the brain parenchyma can induce migration of transplanted cells (Imitola et al., 2004). Thus far there is no report about whether SDF-1 α can induce migration of endogenous generated new neurons to injured cortical tissue following TBI. We explored this in this study by first testing direct injection of SDF-1 α , then by delivery of SDF-1 α to the injured cortex through alginate. In normal animals, when saline was injected into the cerebral cortex, very few DCX-positive new neurons derived from the SVZ were observed moving away from the rostral migratory stream to the site of saline injection. In comparison, injections of SDF-1 α resulted in a considerably higher number of migrating neuroblasts derived from the SVZ to the site of injection. These results confirm the chemotactic properties of SDF-1 α have the same effect *in vivo* as was observed in our *in vitro* migration assays.

Post-TBI introduction of SDF-1 α increases NS/NPCs migration to the site of injury

Following focal tissue injury, the injured cortical tissue directly underneath the impact become necrotic and a cavity is typically formed with time. Formation of this cavity is likely due to several factors, including the mechanical damage caused by the injury itself and the resulting secondary damage as the result of high levels of hypoxia and inflammation. Our previous studies have shown that TBI enhances proliferation of NS/NPCs in the SVZ during the first week following TBI (Sun et al., 2005). Using DCX labeling, we first assessed whether injury-induced new neurons can migrate towards the injury site, we then examined if delivery of SDF-1 α through alginate can enhance the migration of newly generated neurons to the site of injury. As anticipated, TBI alone enhances cell proliferation and neurogenesis in the SVZ of the lateral ventricle and induces some neuroblast migration towards the injury site compared to

uninjured animals. When a SDF-1 α /alginate thread was placed into the injured cortex at 2 days following injury, a higher number of DCX-labeled migrating neurons was observed around the injured cortex compared to CCI only animals. Our data suggest that focal injury alone has a limited effect in inducing cell migration. This is probably due to the low levels of focal production of SDF-1 α /CXCR4 in the TBI animals, as previous study has suggested (Itoh et al., 2009). On the other hand, production of SDF-1 α in stroke models has been shown to increase markedly following injury. This difference is likely due to the varying damage that occurs to neural tissue in stroke animals compared to TBI animals. In stroke victims, hypoxic tissue likely causes upregulation of SDF-1 α production shortly following injury, possibly resulting in the increased neuroblast migration that has been observed in hypoxic-ischemic injury (Robin et al., 2006). As in the ischemic injury model, focal release of higher levels of SDF-1 α , in our case through exogenous delivery via alginate thread, can result in significantly increased migration of newly generated neurons towards the injured area. Collectively, our study suggests that SDF-1 α could be utilized for neuronal replacement therapy for TBI.

CXCR4 Expression surrounding the SVZ and injury cavity increases following TBI

The role of SDF-1 α /CXCR4 in cell migration has been well characterized in many tissues including the CNS during development stage and in ischemic injury (Hagg et al., 2005; Robin et al., 2006; Kojima et al., 2010). To this end, the only study in TBI has reported that SDF-1 α expression is not increased following TBI along with GFAP-positive cells expressing CXCR4 (Itoh et al., 2009). In our study, we found no SDF-1 α staining in any tissue samples, which could be due to the antibody specificity. CXCR4-positive cells were predominantly cells in the SVZ. We also found many CXCR4-positive cells along the corpus callosum and around

the injury cavity in CCI injured animals. These types of positively stained cells were not found in uninjured animals. These changes may account for the increased levels of NSC proliferation and migration in animals affected by TBI. Introduction of SDF-1 α into the injury cavity appeared to have no quantifiable effect on CXCR4 expression. It is likely that expression of the CXCR4 receptor is affected only by the primary and secondary insults of the brain injury and not the subsequent changes in levels of chemokine. Published studies reported that NS/NPCs located in the SVZ of the lateral ventricle of adult rats express both SDF-1 α and CXCR4, suggesting these molecules play an important role in the typical migration of neuroblasts from the SVZ to the olfactory bulb (Tran and Miller, 2003).

Future directions for use of SDF-1 α

Although our study results have suggested that SDF-1 α has the capability to induce NS/NPCs migration both *in vitro* and *in vivo*, additional research must be performed before SDF-1 α can be considered part of any viable treatment protocol for TBI. One initial consideration that warrants further study is the appropriate and most effective dose of SDF-1 α for administration specific to the injury. We found that cells migrated toward SDF-1 α in a dose dependent manner *in vitro*, but the higher doses may prove to have a deleterious effect on neural tissue as the chemokine nature of SDF-1 α could induce a local inflammatory response. Of equal importance would be the determination or establishment of the best method for introduction of the chemokine. Use of alginate threads presents a promising direction for further study due to their ability to hold large amounts of chemokine, the ease of introduction to cortical tissue and the ability of alginate to release SDF-1 α over a sustained period of time. Alginate threads can be cut and formed into various shapes and sizes depending on what is needed at the time.

Considering that SDF-1 α can actually induce large number of cells to move to the injury site, some other questions can be raised. Once the cells reach the site of injury, do these new neurons form synapses with healthy neurons in the surrounding tissue? Do migrating cells differentiate into mostly neurons or glial cells? Can growth factors such as bFGF and EGF be combined with SDF-1 α to enhance cell proliferation and migration? There are no guarantees that increased migration will even be beneficial to neural recovery, and could in fact prove to detrimentally affect recovery. Future studies are needed to answer these questions.

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